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TITLE: Development of Novel Technetium-99m-Labeled Steroids as Estrogen-Responsive Breast Cancer Imaging Agents

PRINCIPAL INVESTIGATOR: Robert N. Hanson, Ph.D.

CONTRACTING ORGANIZATION: Northeastern University
Boston, MA 02115

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Robert N. Hanson, Ph.D.

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14. ABSTRACT

The goal of this project was the preparation and evaluation of new technetium-99m labeled compounds via utilization of their rhenium surrogates. An initial series of rhenium tricarbonyl complexes of estradiol were prepared using Stille coupling methods at the 17-alpha position of estradiol. While the compounds were chemically stable and retain modest affinity for the estrogen receptor compared to estradiol (2-10%), their ability to stimulate or inhibit estrogen function in cells was very low. As a result, the synthetic focus shifted to the 11-beta position of estradiol. Initial examples of 11-beta substituted estradiols were prepared which retained high affinity and potent antiestrogenic activity in cells. Newer rhenium tricarbonyl binding groups were also prepared which had the capability of ligation to the steroidal components using Huisgen [3+2] cycloaddition chemistry. The multi-step process for preparing the steroid component did not provide sufficient material at this time to evaluate the effectiveness of the ultimate steroid-metal binding target compounds.

15. SUBJECT TERMS
Steroidal antiestrogens, tridentate ligands, rhenium tricarbonyl, click chemistry

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1. **Introduction/Statement of Work**

The overall objective of this proposal was to develop new Tc-99m-labeled steroids as potential ER-targeted imaging agents for estrogen responsive breast cancer. To that end we identified 5 specific aims for the period of the award. Those aims were: (1) to prepare an initial series of 17α-E-(pyridyl/histidinyl/bipyridyl) vinyl estradiols to establish synthetic methodology; (2) to screen the initial series of estradiol derivatives for estrogen receptor (ER) binding; (3) to prepare the corresponding Re(CO)3-complexes and evaluate them for ER binding; (4) to prepare and evaluate a second generation of estradiol derivatives and their corresponding Re(CO)3-complexes as ER ligands; and (5) to optimize the complexes and select candidates for radiolabeling/imaging studies.

The previous two interim reports described the progress related to specific aims 1-3. The final report will summarize those results, cover the progress made toward specific aims 4 and 5, and ultimately interpret the significance of the work to date, including a projection regarding where this research should be directed.

2. **Body**

Summary of years 1-2.

During the first two years of the award, the effort was directed toward developing the synthetic chemistry needed to prepare both the steroidal component and the metal tricarbonyl coordinating cores. We initially evaluated the chemistry needed to prepare heterocyclic analogs of the phenyl vinyl estradiols since we would be incorporating pyridyl carboxaldehyde (thiosemicarbazone), bipyridyl and histidinyl groups into the molecule. Our efforts to use vinyl iodides and heteroaryl boronic acids were unsuccessful and so we reverted to vinyl stannanes and heteroaryl halides for the coupling. [Figure 1] The target compounds indicated below were prepared in 18-80% isolated yields and were evaluated for receptor binding and cellular activity. This work has resulted in a manuscript that will be

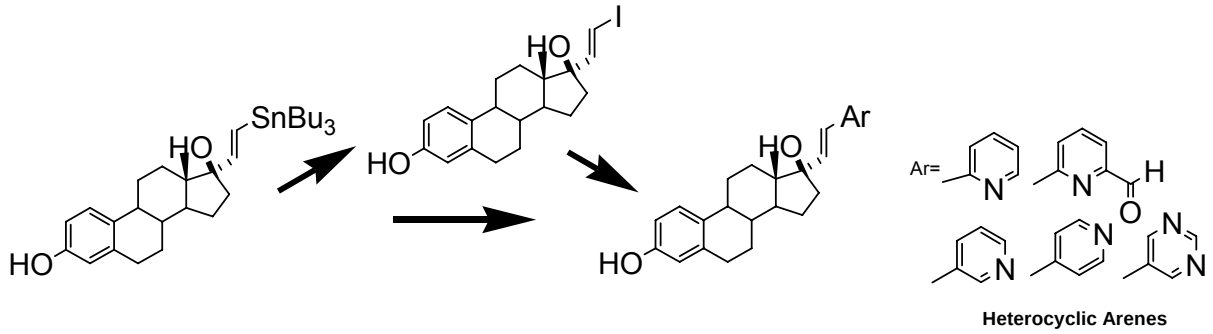


Figure 1. Preparation of heterocyclic vinyl estradiols.

submitted to Journal of Medicinal Chemistry.[See appendix] The chemistry effort also involved developing methods for preparing rhenium tricarbonyl complexes of the pyridyl carboxaldehyde thiosemicarbazone, bipyridyl and histidinyl groups. The work with the benzoylated histidines evolved directly from a prior project related to conjugated phenyl vinyl estradiols. That manuscript (in progress) will be submitted to Bioorganic and Medicinal Chemistry Letters (see appendix). In general, syntheses went very well and gave stable, characterizable complexes. In the bipyridyl series, efforts to couple 5-bromo-2,2'-bipyridine with the stannylvinyl estradiol were unsuccessful, but precomplexation followed by Stille coupling gave the desired compound. This work has resulted in a manuscript that will be submitted to Journal of the American Chemical Society as it represents the first instance of a metallated complex undergoing Stille coupling to a vinyl stannane. [See appendix]

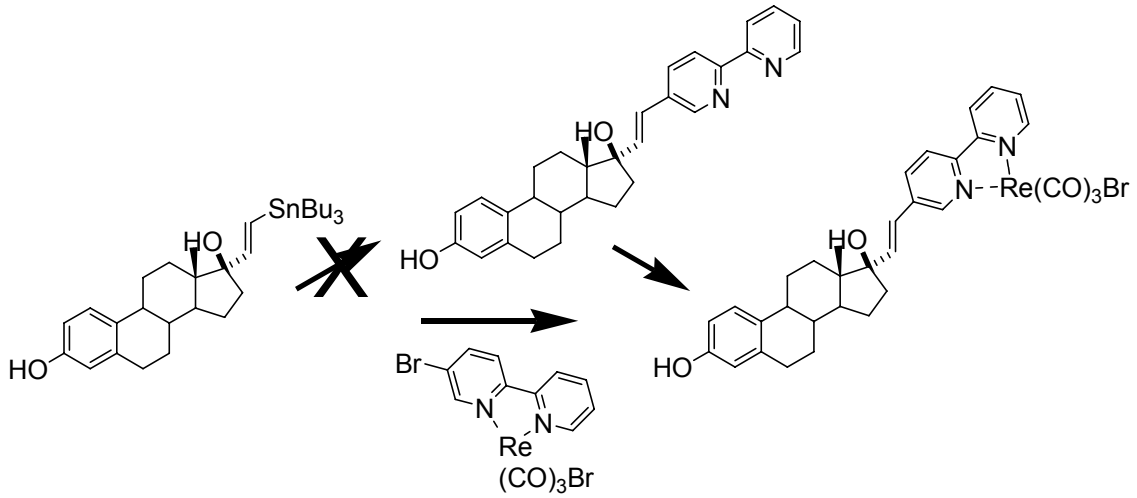


Figure 2. Preparation of Rhenium tricarbonyl complex of bipyridyl vinyl estradiol

Biological evaluation of the compounds prepared during the first two years utilized competitive receptor (estrogen receptor ligand binding domain) binding assays and cellular assays using estrogen stimulated alkaline phosphatase in Ishikawa cells. While all of the new compounds retained significant ER binding affinity, usually 2-8% that of estradiol, the ability of the ligands to either stimulate (agonism) alkaline phosphatase activity or inhibit it (antagonism) was markedly attenuated (<<1% estradiol). This dissociative property was observed with the phenyl vinyl conjugates (benzoylated histidines), the heteroaryl vinyl estradiols and the bipyridyl vinyl estradiols. Either the compounds were not penetrating the cells or they were binding to the receptor in an uncompetitive/unproductive manner. As a result, we felt that further work with these derivatives in which the metal binding group was at the 17α-position was not productive.

Work during year 3.

Our objectives during the third year were to prepare appropriately 11β-substituted estradiols that could be linked/ligated to the requisite rhenium tricarbonyl binding components. This effort related specifically to Specific Aims 4 and 5, development of second generation complexes leading to potential imaging agents. Because we anticipated a convergent approach that would utilize the Huisgen [3+2] cycloaddition reaction, the termini would of necessity be either an alkyne or azide. Our strategy is shown in Figure 3.

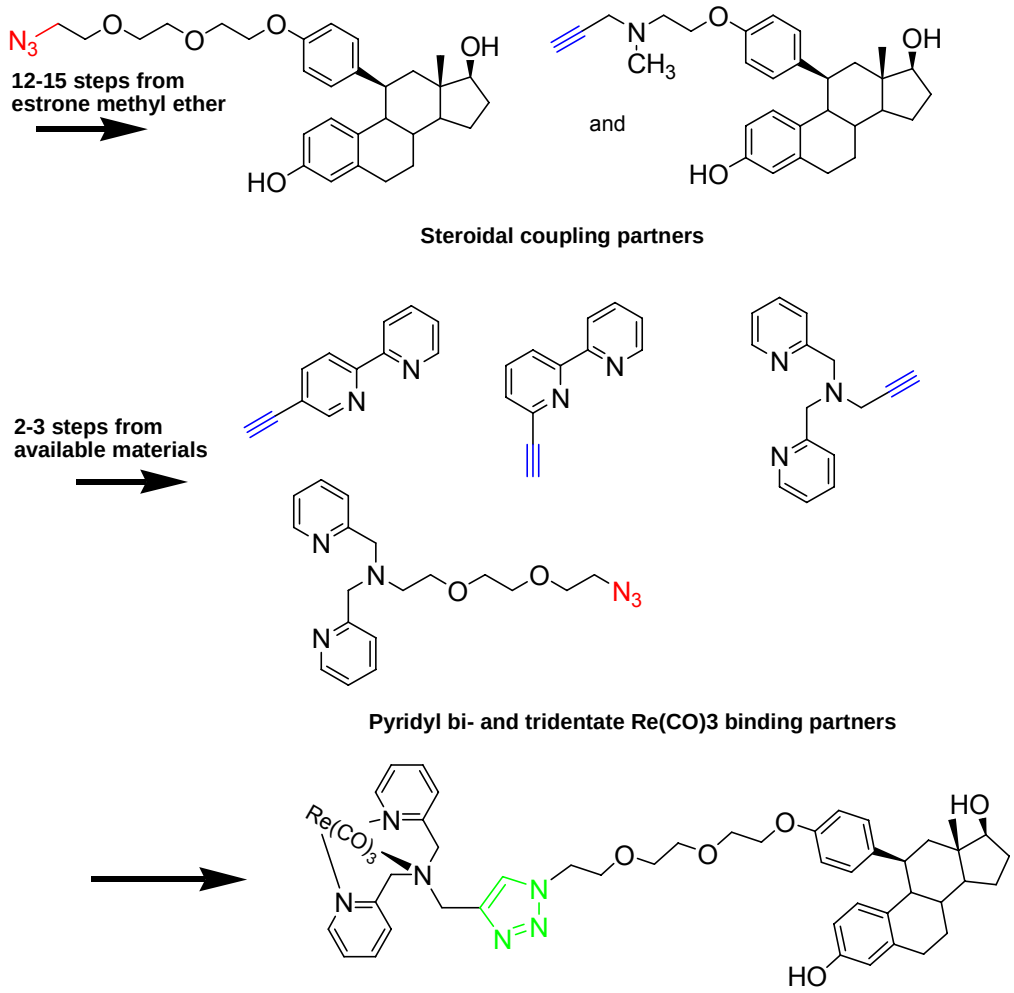


Figure 3. Strategy for preparation of second generation complexes.

The synthesis of the substituted estradiol proceeded from estrone methyl ether to the key diketal of estra-4,9-diene-3,17-dione. Epoxidation of the 5,10-double bond, followed by 1,4-addition of a protected 4-hydroxyphenyl Grignard reagent, and deprotection gave the 11β-(4-hydroxyphenyl)-estra-4,9-diene-3,17-dione. At this point, alkylation with α,ω-ditosyloxy triethylene glycol, followed by displacement with sodium azide, aromatization and reduction of the 17-keto group give the first steroidal component. On the other hand, alkylation with dibromoethane followed by amination with N-methyl propargylamine, aromatization, and reduction of the 17-ketone would give the second steroidal component. In this process, we began with 4 grams of the diketal and ultimately have prepared and characterized 50-100 mg of each steroidal coupling partner. We are in the process of repeating the syntheses on a larger scale to give 200-300 mg of the key coupling materials.

Preparation of the pyridinyl coupling partners was achieved from commercially available or readily synthesized intermediates. Synthesis of the 5/6-bromo-2,2-bipyridines followed by the Sonogashira coupling with trimethylsilyl acetylide and desilylation gave the first two compounds. Reductive amination of picolinyl aldehyde (2-pyridine carboxaldehyde) with propargylamine gave the first of the tridentate ligands while amination with the amino-azido-triethylene glycol gave the second. These reactions proceed more easily and have provided 100-500 mg of key materials for the subsequent reactions. Therefore the chemistry for a variety of potential ligands was established.

The preliminary cyclization reaction for the ligation studies used a model intermediate that we had available. 2-Propargylamino-1,4-naphthoquinone was coupled to the azido-estradiol at a 0.1 mmole scale in a 75% yield using Cu(I) catalysis. Spectroscopic characterization indicated only the expected1,4-substitution product. Therefore the strategy for ligation of the two components was

reasonable. We have also undertaken preliminary cyclization reactions of the ethynyl bipyridine derivatives with model azides (benzyl azide). Again the reactions go well to give the expected 1,4-disubstituted triazoles. In this case, the product will be tridentate rather than bidentate. Subsequent coordination studies with the $Re(CO)_3$ reagents are in progress.

The azido-estradiol and the naphthoquinone ligated compounds were evaluated for ER-LBD binding affinity and for their cellular activity. Binding assays indicate that the two compounds retain high affinity for the estrogen receptor ligand binding domain, 50% and 20% respectively compared to estradiol. More importantly, the two compounds are pure antagonists in the cellular assay with Ki values in the low nanomolar range.

While we have completed key aspects of Specific Aim 4, the intensive nature of the preparation of the steroidal component resulted the need to resynthesis of the key intermediates. We were unable to complete the preparation of the final compounds. Preliminary results suggest that the approach is more likely to be successful than our initial approach. We will continue this work and finish the syntheses and biological assays related to those compounds. Completion of Specific Aim 5 will then involve an evaluation of the results generated to that point and determine whether those results identify a compound sufficiently avid and selective for the estrogen receptor to warrant further study as an imaging agent. We have already begun to develop strategies for either modifying the best of the compounds to improve specific properties or to submit the precursors to collaborators for in vivo studies.

Key Research Accomplishments

- Developed methods for preparation of novel heteroaryl substituted estradiols, including first Stille coupling of metal complex and vinyl stannane
- Improved methods for preparation of unsymmetrical bipyridines and their rhenium tricarbonyl complexes
- Identification of novel class of steroidal antiestrogens that possess high affinity and have incorporated a second biological tag
- Developed novel, convergent approach to metallated, receptor targeted radiopharmaceuticals

Reportable Outcomes.

Manuscripts

Published -none

In preparation (to be submitted Fall 2007)- three

1. **Robert N. Hanson,** Sandra L. Olmsted, Pakamas Tongcharoensirikul, Emmett McCaskill, Karla Gandiaga, David Labaree, and Richard B. Hochberg, Synthesis and evaluation of 17α-E-20-(heteroaryl)norpregn-1,3,5(10),20 tetraene-3,17β-diols[17α- (heteroaryl)vinyl estradiols] as ligands for the estrogen receptor-α-ligand binding domain (ERα-LBD), Journal of Medicinal Chemistry. (In preparation)
2. **Robert N. Hanson**, Rein Kirss, Emmett McCaskill, Edward Hua, Pakamas Tongcharoensirikul, Sandra Olmsted, David Labaree and Richard B. Hochberg, Targeting the Estrogen Receptor with Metal-carbonyl Derivatives of Estradiol, Journal of the American Chemical Society (In preparation)
3. **Robert N. Hanson**, Emmitt McCaskill, Edward Hua, Pakamas Tongcharoensirikul, David Labaree and Richard B. Hochberg, Synthesis of Benzoyl and Benzyl Conjugates of 17α-E-Phenylvinyl Estradiol and Evaluation as Ligands for the Estrogen Receptor-α Ligand Binding Domain, Bioorganic and Medicinal Chemistry Letters (In preparation)

Presentations- National/International Conferences

1. Gandiaga, Karla; Tongcharoensirikul, Pakamas; **Hanson, Robert N..** Preparation of heteroarylvinyl estradiols: Comparison of Suzuki and Stille coupling reactions. Abstracts of

Papers, 229th ACS National Meeting, San Diego, CA, United States, March 13-17, 2005 (2005), MEDI-168.

2. **Hanson, Robert N**.; McCaskill, Emmett. Synthesis and evaluation of a new series of 17alpha-(phenylvinyl) estradiol conjugates as probes for the estrogen receptor-alpha ligand binding domain (ERalpha-LBD). Abstracts of Papers, 229th ACS National Meeting, San Diego, CA, United States, March 13-17, 2005 (2005), MEDI-170.
3. Hua, Edward Y.; Labaree, David C.; Hochberg, Richard B.; **Hanson, Robert N**.. Synthesis and evaluation of Estradiol-PEG-DNA alkylation agents using click chemistry. Abstracts of Papers, 232nd ACS National Meeting, San Francisco, CA, United States, Sept. 10-14, 2006 (2006), MEDI-157
4. Olmsted, Sandra; **Hanson, Robert N.**; Tongcharoensirikul, Pakamas; McCaskill, Emmett; Hochberg, Richard B.; Labaree, David C. Synthesis and evaluation of 17-alpha-heteroarylvinyl estradiols as ligands for the estrogen receptor ligand binding domain (ER-LBD). Abstracts of Papers, 233rd ACS National Meeting, Chicago, IL, United States, March 25-29, 2007 (2007), MEDI-373.
5. Hanson, Robert N. Technetium/rhenium tricarbonyl labeled and fluorinated estradiols for estrogen receptor imaging: New Variations. Abstracts of Papers, 234th ACS National Meeting, Boston, MA, United States, August 19-23, 2007, NUCL-6.

Funding applied for based on this research:
Two proposals have been submitted to the BCRP 2007 related to the use of the 11-beta position as the optimal site for incorporating either targeting or imaging groups.

Personnel supported all or in part during this award:
Northeastern University
Robert N. Hanson,Ph.D.-P.I.
Rein Kirss, Ph.D.- Co-.I.
Emmett McCaskill, Ph.D.-Research Associate
Pakamas Tongcharoensirikul, Ph.D.-Research Associate
Edward Hua, M.S.- Graduate Student (Ph.D. 2007)

Yale University School of Medicine
Richard B. Hochberg, Ph.D.-Co.-I.
David Labaree, Ph.D.-Research Associate

Conclusions:
The development of imaging agents for the estrogen receptor requires the consideration of at least three factors:

1. Successful preparation of ER ligands that retain high affinity. We have determined that for ER-targeting, the 11-beta position is the optimal site for functionalization, however, its utilization requires skill in multi-step organic synthesis. The 17-alpha position, typically used because it is easy to access, **is not** the appropriate site for conjugation.
2. Incorporation of the metal binding groups must allow the metal to be complexed **at the last step.** We have identified new metal carbonyl chelating moieties and variations of previously known materials, but we had clarified issues that must be address in linking them to the ER-targeting groups. This involves relatively simple but necessary chemical modifications. A convergent synthesis using "click" chemistry is appropriate for radiopharmaceuticals.
3. High affinity for the target receptor is **necessary but not sufficient.** We have found that relatively small changes in structure alter the biological properties significantly. Many of the initial series had reasonable affinities but either did not penetrate cells or gave discordant biological results. This was

the **major observation** that necessitated the change from 17-alpha substituted estradiols to the 11-beta substituted estradiols.

What our studies suggest regarding future research can be summarized briefly. Steroid-based, metallated radiopharmaceuticals for targeting the estrogen receptor containing tissues may be successful if they utilize the 11-beta position for incorporation of the metal binding group at a distance that will be external to the ligand binding pocket. Such approaches are structure-based, intensive in synthetic skill, and utilize multiple biological validation methods. Given that there currently exist no clinically available radiotracers that can successful detect ER-positive breast cancer- primary, metastatic or recurrent- this research is still important. Multiple strategies that address these themes should be encouraged.

References-None

Appendix: W81XWH-04-1-0544
Development of Novel Technetium-99m-Labeled Steroids as Estrogen-Responsive Breast Cancer Imaging Agents
P.I.- Robert N. Hanson, Ph.D.
Copies of Manuscripts in Preparation (1-3)

1. Current version July 10, 2007

Synthesis and evaluation of 17α-E-20-(heteroaryl)norpregn-1,3,5(10),20 tetraene-3,17β-diols[17α- (heteroaryl)vinyl estradiols] as ligands for the estrogen receptor-α-ligand binding domain (ERα-LBD)

Robert N. Hanson, Sandra L. Olmsted, Pakamas Tongcharoensirikul, Emmett McCaskill, Karla Gandiaga, David Labaree, and Richard B. Hochberg

Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, MA 02115

Department of Chemistry, Augsburg College, 2211 Riverside Avenue, Minneapolis. MN 55454

Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520

Abstract:

A series of 17α- (heteroaryl)vinyl estradiols was prepared to evaluate the influence of heteroatom on the affinity of estrogenic ligands for the estrogen receptor-alpha ligand binding domain ERα-LBD). The compounds were synthesized using Stille coupling when Suzuki coupling proved to be problematical. The products demonstrated reduced binding affinity compared to the parent 17α-E-phenylvinyl estradiol, but the binding was relatively unaffected by the heteroatom present within the heteroaryl ring. The influence of the heteroatom was also evident in the efficacy of the compounds as the thienyl derivatives **3f,g** were more potent than

either the pyridyl **3b-d** or pyrimidinyl **3e** analogs. The results indicate that a subtle interplay of interactions between the ligands and the receptor residues influences the biological response. Overall, the highest ERα-LBD binding affinity and estrogenic activity were observed with the parent compound **3a,** suggesting a careful balance in the electron density of the aromatic ring is preferred.

Introduction:

The estrogen receptor (ER) is a member of the nuclear receptor (NR) superfamily of transcription factors, a group that mediates a wide variety of physiological and developmental processes.[1-3] Because inappropriate or over-expression of ER is associated with a number of endocrine disorders, such as breast, endometrial and ovarian cancer, and osteoporosis, chemical modulation of the ER-regulated pathways is a critical clinical objective. Recent reviews have described the structure of the ER, including its subtypes, and the general mechanism by which binding of the endogenous ligand and its steroidal and nonsteroidal analogs initiate the events leading to transcription (agonist responses) or modulation of transcription (antagonism/selective ER modulation). [4-11] While many individual steps are involved in the overall process, the initial binding of the ligand to the (apo)receptor to generate a stable complex constitutes the key step that defines the subsequent events. Virtually all subsequent biological responses are influenced by the receptor-ligand complex conformation induced by this interaction. Analysis of the structures of ligand-ER-LBD complexes has provided significantly enhanced understanding of these interactions, yet the static nature of crystal stucures leaves many questions unanswered.[12-15] Based upon this premise, research efforts to characterize that initial step remain important for understanding how the subsequent biological consequences are generated.

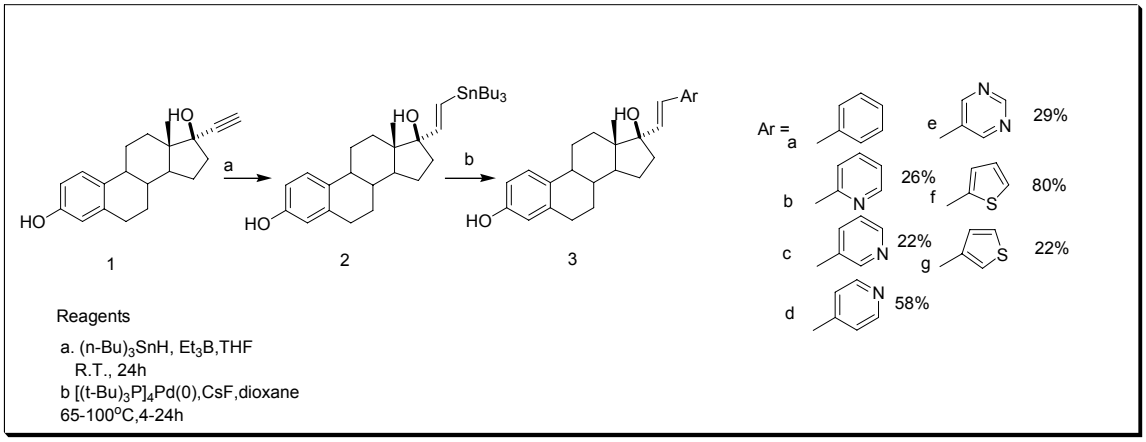
Our research program has focused on probing the topology of the ERα-LBD through the preparation and evaluation of systematically modified derivatives of estradiol. [16-23] The structural probes would permit enhanced insight into the influence of physicochemical properties in modulating receptor affinity, selectivity and efficacy. Most of our initial work has concentrated on the 17α-position through the use of modifications of the phenylvinyl moiety. [18-20,22,23] These studies demonstrated that, in general, small substituents on the 17α-phenylvinyl group were well tolerated (high affinity), but the binding was strongly influenced by stereochemistry around the carbon-carbon double bond and particularly by the position and nature of the moiety on the phenyl

group. However, these binding potencies were largely, but not absolutely, translated to *in vivo* activity. Of particular note, all of the simple phenylvinyl derivatives, regardless of E-/Z- stereochemistry or ortho-, meta-, para-substitution, were agonists. Molecular modeling studies, coupled with x-ray crystallographic analysis of one of the compounds complexed to ERα-LBD, indicated that the ligand and the receptor induce mutual adaptive responses to generate an agonist conformation for all of the simple, mono-substituted derivatives. [24] Because even small substituents induce changes within the protein structure, we decided to eliminate the pendant groups and modify the nature of the aromatic moiety. Such modifications should reduce the steric factors while providing changes in the electronic character of the aromatic ring. This paper describes the effects of such modifications on the affinity and efficacy of the 17α-E-heteroarylvinyl estradiols.

Results and Discussion:

Synthesis of 17α-E-heteroarylvinyl estradiols

The synthetic strategy for preparing the target compounds is shown in Scheme 1.



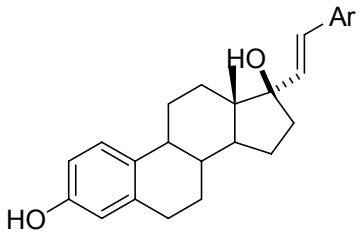
Scheme 1.Synthesis of (hetero)aryl vinyl estradiols **3a-g**

Initially we proposed preparing the target compounds via the Suzuki coupling between 17α-E-iodovinyl estradiol and the corresponding heteroaryl boronic acid. The rationale for this method was the apparent ease of the Suzuki coupling reaction, the commercial availability of the aryl boronic acids and their lower toxicity. Unfortunately, this method proved to be inconsistent and unreliable, giving only some of the desired products in very low yields (<5%) along with homo-coupling of the iodovinyl estradiol as the major product. This observation led us to use our previously established Stille coupling strategy. Coupling 17α-E-tri-n-butylstannylvinyl estradiol **2** with the corresponding bromo/iodoheteroarene gave the desired heteroarylvinyl estradiols **3b-g**, in acceptable overall yields (18-80%) with minimal by-products. In some cases the product was accompanied by the homocoupled vinyl estradiol dimer **4**. ^{1}H-NMR confirmed the anticipated E-stereochemistry (J= 16 Hz) while the chemical shifts of the C_{20},C_{21}-protons reflected the electron donating/withdrawing character of the heteroarene. The observed chemical shifts ranged from 6.41-6.51 and 6.67-6.77 δ for the electron rich thienyl derivatives to 6.56-6.69 and 6.86-7.18 δ for the electron-deficient pyridyl and

pyrimidinyl and derivatives. Chromatography demonstrated that the nitrogen-containing heterocyclic arylvinyl estradiols were significantly more polar than the corresponding phenyl- and thienylvinyl estradiols.

Biological Evaluation:

The new compounds and the parent 17α-E-phenylvinyl estradiol **3a** were evaluated for estrogen receptor binding affinity using the ERα-LBD derived from *E. Coli* and for efficacy using induction of alkaline phosphatase in Ishikawa cells.[25-27] The results are shown in Table 1. As the data indicate, the parent compound, previously characterized has binding and efficacy that are approximately 10% that of estradiol. Introduction of one nitrogen (2-/3-/4-pyridyl), two nitrogens (5-pyrimidinyl) or a sulfur (2/3-thienyl) into the ring produced a significant (2.5-10-fold) reduction in the RBA values. The effect of heteroatom substitution on efficacy was different as only the thienyl derivatives exhibited significant stimulatory activity in this assay (relative stimulatory activity [RSA] = 2.5%) whereas the aza-heterocylic derivatives were essentially inactive (RSA = 0.1-0.4%).



AR =	RBA+/-S.D.	RSA+/-S.D.
phenyl-**3a**	10.3 ± 2.9	9.5 ± 2.5
2-pyridyl-**3b**(SLO-1291)	1.5 ± 0.8	0.1 ± 0.07
3-pyridyl-**3c**(SLO-0562)	4.0 ± 1.0	0.25 ± 0.07
4-pyridyl-**3d**(SLO-1082)	2.5 ± 1.0	0.1 ± 0.1
5-pyrimidinyl-**3e**(SLO-0772)	0.8 ± 0.3	0.4 ± 0.2
2-thienyl-**3f**(SLO-0370)	2.7 ± 1.2	2.5 ± 0.1
3-thienyl-**3g**(SLO-0459)	2.9 ± 0.4	2.5 ± 0.8

Table 1. Relative binding affinity and relative stimulatory activity of heterarylvinyl estradiols compared to estradiol. RBA estradiol = 100% and RSA estradiol = 100%

In previous studies we observed that the nature and position of substituents on the phenyl ring had a significant effect on the ability of the ligand to bind to the ERα-LBD. In this study we selected heterocyclic arenes that are essentially isosteric to

benzene and differ primarily in their electronic character. The two thienyl derivatives are more electron rich than the phenyl analog while the pyridyl derivatives are electron deficient and have a weakly basic nitrogen (lone pair) oriented toward the ortho (2-), meta- (3-) or para (4-) position.[28,29] The pyrimidinyl derivative is even more electron deficient and has two more weakly basic nitrogens symmetrically oriented toward the meta- (3-,5-) positions. One of the effects of substitution that was observed during the purification of the products was the increased polarity of the nitrogen-containing heterocyclic derivatives. Significantly more polar solvents were required to elute the products from the column compared to the thienyl and phenyl analogs, probably related to the hydrogen bond accepting properties of the ring nitrogen(s). This property would also carry over to the ERα-LBD where such interactions may also be present. The binding pocket into which the arylvinyl group is inserted is bounded by three methionine residues (Met-342,-343,-421) and one phenylalanine (Phe-425).[24] Repulsive interactions between the electron pairs of the heteroarenes and those present in methionines may partially explain the reduced binding affinity observed for the new derivatives. In addition, interactions between the π-electrons of the heteroarenes and the adjacent phenylalanine are possible but not likely given the distance and orientation of the two aromatic rings. However, it is more difficult to use this reasoning to explain why the thienyl but not the pyridyl or pyrimidinyl derivatives would retain agonist properties. In previous studies we have seen that the introduction of an 11β-methoxy group has little effect on overall binding affinity yet dramatically increases agonist activity, presumably by influencing the stability of receptor-coactivator complexes. In this case, we may be observing the opposite effect where the aza-arenes subtly weaken the internal forces that favor coactivator binding, without generating major changes in ligand binding affinity.

In summary, we have demonstrated the preparation of a series of heterocyclic analogs of the parent 17α-phenylvinyl estradiol using Stlle coupling methods. Biological assays indicated that the introduction of the heteroatom had the general effect of reducing binding affinity, however, the efficacy for the aza-arene derivatives was dramatically reduced compared to the thienyl and the parent phenyl derivatives. The results suggest that interactions between the electron pairs on the aryl group and the surrounding peptide residues have clear, but unpredictable effects on biological properties. Because understanding these interactions is important in relating chemical structures with biological responses within the estrogen receptor field, further studies are in progress.

Experimental

General Methods. All reagents and solvents were purchased from Aldrich or Fisher Scientific. THF and toluene were distilled from sodium/benzophenone. Reactions were monitored by TLC, performed on 0.2 mm silica gel plastic backed sheets containing F-254 indicator. Visualization on TLC was achieved using UV light, iodine vapor and/or phosphomolybdic acid reagent. Column chromatography was performed on an Argonaut Flashmaster using prepacked Isolute silica gel columns (Biotage). Melting points were determined using an Electrotherm capillary melting point apparatus and are uncorrected. NMR spectra chemical shifts are reported in parts per million downfield from TMS and referenced either to TMS internal standard for deuterochloroform or deuteroacetone solvent peak. 1H-, 13C-NMR spectra, HRMS and elemental analyses (Desert Analytics, Tucson, AZ)are provided.

General Synthetic Method. Example -17α-E-(3-Pyridyl)-vinyl estradiol 3c.
17α-E-tri-n-butylstannylvinyl estradiol **2** (0.50 mmol, 0.293 g) , 3-iodopyridine (1.50 mmol, 0.310 g), dried cesium fluoride (0.40 g), and 25 mg bis (tri-t-butylphosphine)palladium (0) were evacuated and purged with argon four times. Dry dioxane (3 mL) was added, the mixture was sealed under an argon atmosphere and heated at 80°C until the reaction was complete (monitored by TLC).

The hot reaction mixture was filtered and the residue was washed with acetone. The filtrate was evaporated to dryness and the product was purified by automated flash chromatography on silica gel using hexane-ethyl acetate (gradient) as the eluent. The fractions containing pure product were combined and evaporated to yield 34 mg (0.09mml, 18% yield). The product was characterized by ^{1}H-, ^{13}C-NMR, HRMS, and elemental analysis. Characterization of the less polar major component identified the material as the vinyl estradiol homodimer **4**.

17α-E-(2-Pyridyl)-vinyl estradiol 3b.
17α-*E*-(2-pyridinyl)-vinyl estradiol (SLO-1291)
Yield: 53 mg, 0.148 mmol, 26% of theory.
NMR: 8.486, 8.478 (d, 1H); 7.747 (d, 1H); 7.629; 7.618, 7.614; 7.602, 7.598 (split t, 1H); 7.298, 7.283 (d, 1H); 7.192, 7.160 (d, 1H); 6.680, 6.648 (d, 1H); 7.090, 7.081; 7.075, 7.066 (dd, 1H); 7.028, 7.011 (d, 1H); 6.680, 6.648 (d, 1H); 6.483, 6.477; 6.466, 6.461 (split d, 1H); 6.431, 6.427 (split s, 1H)

17α-E-(4-Pyridyl)-vinyl estradiol 3d.
17α-*E*-(4-pyridinyl)-vinyl estradiol (SLO-1082)
Yield: 160 mg, 0.427 mmol, 58% of theory.
NMR: 8.461, 8.458; 8.452, 8.449 (split d, 2H); 7.327, 7.314 (d, 2H); 7.033, 7.016 (d, 1H); 6.877,6.845 (d, 1H); 6.617, 6.585 (d, 1H); 6.489, 6.484; 6.473, 6.468 (split d, 1H); 6.434, 6.430 (split s, 1H)

17α-E-(5-Pyrimidinyl)-vinyl estradiol 3e.
17α-*E*-(5-pyrimidinyl)-vinyl estradiol (SLO-0772)
Yield: 58 mg, 0.154 mmol, 29% of theory.
NMR: 8.969 (s, 1H); 8.870 (s, 2H); 7.060, 7.033 (d, 1H); 6. 879, 6.825 (d, 1H); 6.614, 6.560 (d, 1H); 6.533, 6.523; 6.496, 6.473 (split d, 1H); 6.473 (s, 1H)

17α-E-(2-thienyl)-vinyl estradiol 3f.
17α-*E*-(2-thienyl)-vinyl estradiol (SLO-0356 and -0370)
Yield: 0.163 mg, 0.428 mmol, 80% of theory.
NMR (500 mHz): 7.858 (s, 1H, Ar-OH); 7.264, 7.248 (d, 1H); 7.090, 7.061 (d, 1H); 7.025,7.017, (d, 1H); 6.991, 6.979; 6.974, 6.963 (split d, 1H); 6.796, 6.744 (d, 1H); 6.595, 6.585; 6.566, 6.557 (dd, 1H); 6.523, 6.514 (d, 1H); 6.434, 6.381 (1, 1H)

17α-E-(3-thienyl)-vinyl estradiol 3g.
17α-*E*-(3-thienyl)-vinyl estradiol (SLO-0459 and -0476)
Yield: 37 mg, 0.097 mmol, 22% of theory.
NMR: 7.954 (s, 1H, Ar-OH); 7.406, 7.400; 7.396, 7.390 (dd, 1H); 7.355,7.353; 7.345,7.343 (split d, 1H); 7.303,7.297 (d, 1H); 7.097, 7.079 (d, 1H); 6.683,6.650 (d, 1H);6.625, 6.619; 6.607, 6.603 (dd, 1H); 6.558, 6.553 (d, 1H); 6.525, 6.494 (d, 1H)

Competitive Binding to the Human Estrogen Receptor alpha Ligand Binding Domain (ERα-LBD). Binding to ERα-LBD was measured by displacement of [3H]E2 (~1 nM) in incubations performed at room temperature overnight with lysates of *Escherichia coli* in which the LBD of human ERα (M250-V595) is expressed [25,26]. For the assay, the lysates were incubated with nonradioactive E2 and the arylvinyl estradiol derivatives over a range of concentrations from10-6 to 10-12 M. Binding affinity (RBA) relative to estradiol was determined by analysis of the binding curves by the curve-fitting program Prism (GraphPad Software, Inc., San Diego, CA 92130). The results are averages of eight separate experiments performed in duplicate.

Estrogenic Potency in Ishikawa Cells The estrogenic potency of the17α-substituted estradiol derivatives was determined in an estrogen bioassay, the induction of AlkP in human endometrial adenocarcinoma cells (Ishikawa) grown in 96-well microtiter plates as has been previously reported [27]. The cells are grown in phenol red free medium with estrogen depleted (charcoal stripped) bovine serum in the presence or absence of varying concentrations of the steroidal derivatives over a range of 6 log orders. After three days, the cells are washed, frozen, thawed and the incubated with 5 mM p-nitrophenyl phosphate, a chromogenic substrate for AlkP enzyme, at pH 9.8. To ensure linear enzymatic analysis, the plates are monitored kinetically for the production of p-nitrophenol at 405 nm. The relative stimulatory activity (RSA) is determined by analysis with the curve fitting program Prism (GraphPad Software, Inc., San Diego, CA 92130). Each compound was analyzed in at least three separate experiments performed in duplicate.

Acknowledgments.

We are grateful for support of this research through grants from the National Institutes of Health [PHS 1R01 CA81049 (R.N.H.) and PHS 1R01 CA 37799 (R.B.H.)], the U.S.Army Breast Cancer Research Program [DAMD 17-00-1-00384 and W81HW-04-1-0544(R.N.H.)] .

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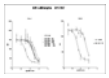
Supporting Information
Elemental analyses and/or HRMS for new compounds.
1H-NMR spectra for new compounds.

Compound #	Mol formula	Calc'd	Found	HRMS
3b	C25H29NO2	C=79.96;H=7.78;N=3.73	C=79.32; H= 7.37; N=3.53	M+1= 375.2275
3c	C25H29NO2 (RNH-IX-14)	C=79.96;H=7.78;N=3.73	C= 76.77; H= 7.37; N = 3.17 C= 79.19;H = 7.44; N = 3.60	M+1= 376.2295
3d	C25H29NO2	C=79.96;H=7.78;N=3.73	C = 74.12; H = 7.56; N = 3.36	M+1 = 376.2275
3e	C24H28N2O2	C=76.56; H=7.50;N=7.44	C=76.98; H=7.36; N=7.25	M = 376.2180
3f	C24H28SO2	C=75.75; H= 7.42:	C=75.93; H= 7.48;	M= 380.1802
3g	C24H28SO2	C=75.75; H= 7.42:	C= 75.48; H= 7.69	M= 380.17872



17α-E-(2,3,4,5,6-pentafluorophenyl)-vinyl estradiol 3h.
17α-*E*-(pentafluorophenyl)-vinyl estradiol (SLO-0978)
Yield: 15 mg, 0.032 mmol, 5% of theory.
NMR (CD_3OD): 7.086, 7.069 (d, 1H); 6.885, 6.853 (d, 1H); 6.571, 6.538 (d, 1H); 6.554, 6.549; 6.538, 6.531 (split d, 1H); 6.496, 6.490 (split s, 1H)

2.

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Targeting the Estrogen Receptor with Metal-carbonyl Derivatives of Estradiol

Robert N. Hanson, Rein Kirss, Emmett McCaskill, Edward Hua, Pakamas Tongcharoensirikul, Sandra Olmsted, David Labaree and Richard B. Hochberg

Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, MA 02115
Department of Chemistry, Augsburg College, 2211 Riverside Avenue, Minneapolis, MN 55454
and Department of Obstetrics/ Gynecology and Reproductive Sciences, Yale University School of Medicine, 333 Cedar Street, New Haven, Ct 06520

Abstract: We have designed and synthesized a novel rhenium tricarbonyl derivative of estradiol as a potential breast cancer imaging agent based upon the current understanding of the steroidal ligand-estrogen receptor binding process. Although simple 17α-(hetero)arylvinyl derivatives of estradiol can be prepared via palladium(0) catalyzed Stille coupling reaction, the incorporation of the rhenium tricarbonyl group is more challenging. In this study we evaluated metallated and nonmetallated approaches to the preparation of rhenium tricarbonyl substituted bipyridyl vinyl estradiol. The synthesis constitutes the first report of a Stille coupling between a metallated complex and a vinylstannane. The final product retains significant estrogen receptor binding properties suggesting that further structural modifications of the ligand known to enhance affinity may lead to estrogen receptor-selective breast cancer imaging agents.

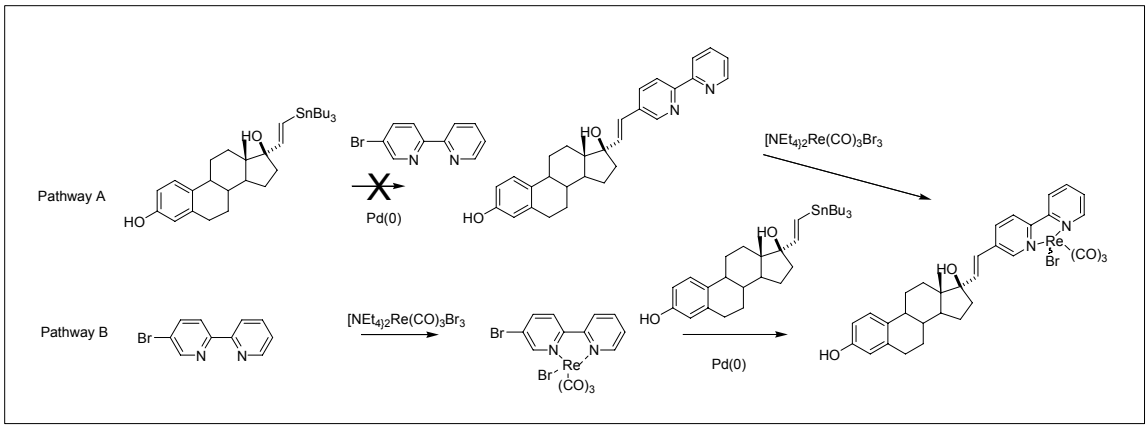
1. Introduction:

Detection of estrogen-responsive breast cancer using radiolabeled estrogens remains a major diagnostic goal of nuclear medicine in spite of over 25 years of research. Although initial efforts in the field focused on radio-iodinated derivatives of estradiol, which demonstrated considerable success at the pre-clinical stage [1], most of the later studies utilized [F-18]-estrogens, primarily because of the superior imaging and radiation dosimetry properties associated with the [F-18]-positron-emitting radionuclide [2]. However, the need for an efficient onsite cyclotron to generate the radionuclide in high specific activity limits the availability of such radiopharmaceuticals, thereby stimulating the search for alternatives. Technetium-99m is a readily available nuclide with highly desirable physical properties (a 6 h half life, 140 keV gamma ray emission energy and generator production), however, its chemical (metallic) properties limit its incorporation into many small bio-organic molecules. Initial efforts to prepare small Tc-chelating groups that did not significantly alter the physicochemical and biological properties of estrogenic ligands were unsuccessful [3]. Smaller, more stable cyclopentadienyl Tc/Re tricarbonyl complexes retained many of the desired biological properties, however, the radiolabeled compounds were not accessible in appropriate time scales and overall radiochemical yields.[4,5] Recent studies, primarily through use of its rhenium surrogate, indicate that Tc-99m tricarbonyl derivatives are readily

prepared in an aqueous environment, can be coordinated to many small molecules and still retain the desired biological properties.[6] For example, recent studies by Arterburn, et al., using 17-α-substituted estradiol-pyridin-2-yl hydrazine conjugates suggested that such complexes may be designed in which the requisite ER-binding is not sacrificed. [7]. In this manuscript we report the preparation, via an innovative approach, of a new rhenium tricarbonyl derivative of estradiol that retains significant affinity for the estrogen receptor-alpha subtype accompanied by altered efficacy.

In developing the rhenium tricarbonyl derivative of estradiol we utilized our experience with both the synthesis of 17α-arylvinyl estradiols and chelating properties of 2,2'-bipyridines. Our ongoing research directed toward ER-ligands demonstrated that the ligand binding pocket (LBP) complementary to the 17α-position of estradiol can accommodate the substituted phenylvinyl groups with affinities comparable to the endogenous ligand, estradiol [8]. Our synthetic approach for preparing these derivatives utilized Pd(0) coupling of the stannylvinyl estradiol with the requisite substituted aryl halide. Subsequent Stille coupling of tri-n-butylstannylvinyl estradiol with heteroaryl halides generated a series of derivatives that displayed moderate affinity for the estrogen receptor ligand binding domain (ER-LBD), roughly comparable to the phenylvinyl derivative.[9]

Bipyridines are capable of coordinating a wide variety of transition metals, including rhenium tricarbonyl species [10]. Our initial strategy for demonstrating proof of principle therefore involved preparing the unsymmetrical 5-bromo-2,2'- bipyridine, and coupling it to the stannylvinyl estradiol via the Stille reaction. The resulting bipyridyl vinyl estradiol intermediate could ultimately be labeled with the corresponding rhenium tricarbonyl reagent (Pathway A).



Scheme 1. Routes to the $Re(CO)_3$-bipyridyl-vinyl estradiol complexes

Alternatively, the 5-bromo-2,2'-bipyridine could first undergo chelation with the rhenium tricarbonyl reagent followed by Stille coupling to give the final compound (Pathway B). The first route would require that the bipyridine not undergo transchelation by the palladium catalyst, while the second would require the metallated bipyridine to be a successful coupling partner. There was no literature precedent for either pathway that proceeds through Stille coupling of a vinylstannane.

2. Results and Discussion:

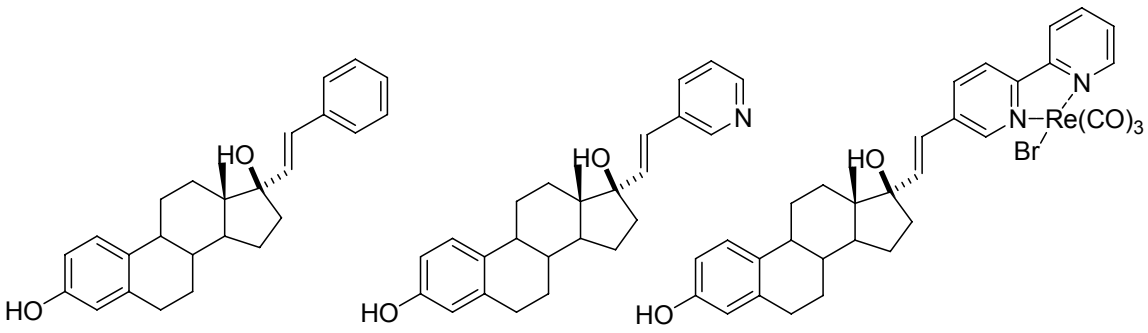
The preparation of the stannylvinyl estradiol proceeded via our established method (8a,8c) while the unsymmetrical 5-bromo-2,2'-bipyridine was generated by Stille coupling of 2, 5-dibromopyridine with 2-trimethylstannylpyridine.(11) The rhenium derivative, 5-bromo-2, 2'-bipyridine)$Re(CO)_3Br$, was obtained by reaction of 5-bromo-2, 2'-bipyridine and $[NEt_4]_2[Re(CO)_3Br_3]$ **(**12**)** in methanol. Although synthesis of the model 3-pyridylvinyl estradiol proceeded without difficulty, our efforts to couple the stannylvinyl estradiol with 5-bromo-2,2'-bipyidine proved to be unsuccessful, using a variety of Stille coupling procedures. Changes in catalyst [$\{(C_6H_5)_3P\}_4Pd(0)$, Pd_2dba_3-$(C_6H_5)_3P$, $\{(t\text{-}C_4H_9)_3P\}_4Pd(0)$], solvent (THF, 1,4-dioxane, toluene) and temperature (R.T., 60°C, reflux) did not yield detectable quantities of coupled product. The reasons behind the failure of the Stille coupling in the latter case are difficult to determine. Successful Stille coupling of bromobipyridines with arylstannanes and heteroarylstannanes in good yield have been reported. [13] Coupling of Bu_3Sn-functionalized Troger's base with 2-bromopyridine, however, required high temperatures (100°C) and proceeded in yields ranging from 0-64% depending on the catalyst system used. [14] Lower yields were ascribed to catalyst decomposition. We believe that the lower reactivity of vinylstannanes compared to arylstannanes and catalyst degradation may have contributed to the failure of the reaction between 5-bromo-2, 2'-bipyridine and the stannylvinyl estradiol.

Stille coupling of 5-bromo-2, 2'bipyridine)$Re(CO)_3Br$ and the stannylvinyl estradiol proved to be more successful with the desired product obtained in 30% isolated yield. The $Re(CO)_3$ fragment in 5-bromo-2, 2'bipyridine)$Re(CO)_3Br$ acts as an electron withdrawing group when coordinated to the bromobipyridine ligand, promoting oxidative addition of the C-Br bond to Pd(0). [15] Electron-withdrawing groups on aryl groups in Pd-aryl intermediates also promote reductive elimination of C-C bonds in the Stille reaction. [16] Both of these effects may be contributing to the greater success of the cross-coupling reaction between 5-bromo-2, 2'bipyridine)$Re(CO)_3Br$ and stannylvinyl estradiol compared to the reaction of 5-bromo-2, 2'-bipyridine. The use of a polyfunctional and less reactive stannane (aryl > vinyl), as well as the limited solubility of the 5-bromo-2, 2'bipyridine)$Re(CO)_3Br$

in non-protic solvents that are compatible the Stille coupling, may also be contributing to lower yields for the reaction of 5-bromo-2, 2'bipyridine)$Re(CO)_3Br$ and stannyl vinyl estradiol than observed in Stille couplings of bromobipyridine with arylstannanes cited earlier.

The synthesis of the vinyl-bipyridine estradiol-$Re(CO)_3$ complex adds to the limited examples of Stille coupling between transition-metal coordinated arenes or aromatic heterocyclic ligands. Stille coupling of (η^6-chlorobenzene)$Cr(CO)_3$ and (η^6-p-chloroanisole)$Cr(CO)_3$ with 2-tributylstannyl-thiophene yielded the cross-coupled products in 55 and 40%, respectively [17]. Cationic (η^6-chloroarene)$Mn(CO)_3^+$ complexes readily reacted with $Pd(PPh_3)_4$ to form a stable intermediate that was inert toward further reaction, an observation attributed to the strong electron withdrawing effect of the $Mn(CO)_3^+$ group. [18] The $Cr(CO)_3$ also acts as an electron withdrawing group when coordinated to arene ligands. The synthesis of the vinyl-bipyridine estradiol-$Re(CO)_3$ complex is unique in using a vinylstannane rather than an arylstannane.

The receptor binding affinity of the $Re(CO)_3$-bipyridyl-vinyl estradiol complex for the ERα-LBD was determined by radiometric assays with [H-3] estradiol, expressed as relative binding affinity (RBA) compared to estradiol (100%)[19,20]. The nature of



RBA =	10.3 +/- 2.9%	4.0 +/- 1.0 %	4+/- 0.1%
RSA =	9.5 +/- 2.5%	0.25 +/- 0.07 %	0.4 +/- 0.1%

Figure 1. Relative Binding Affinity (RBA) and Stimulatory Activity (RSA) values for the phenylvinyl, pyridylvinyl and $Re(CO)_3$-bipyridylvinyl estradiols.

the aryl group had only a modest effect on the binding affinity of the compound for the ERα-LBD. As previous studies have shown, introduction of the terminal phenyl ring reduced receptor binding compared to estradiol,[8] however, replacement of the phenyl ring by the isosteric pyridyl group did not dramatically reduce the RBA value, 4.0% versus 10.3%. As the binding results indicate, further modification by appending the second pyridyl ring para to the first and introducing the metal carbonyl moiety had no additional effects on the RBA value. This observation is similar to that reported by Arterburn, et al., [7a,b] and Gabano, et al.[7c] with their complexes and suggests that the ERα-LBD can accommodate significant structural diversity, including heterocyclic and metallated groups at the 17α-position.

To determine the functional response of this complex we used the ligand-induced alkaline phosphatase activity in ovarian adenocarcinoma (Ishikawa) cells, expressed as relative stimulatory activity (RSA) compared to estradiol (100%).[2] The parent compound, 17α-phenylvinyl estradiol, demonstrated an RSA value that was comparable to its observed RBA values (10.3 vs 9.5%). The 3-pyridyl analog and the $Re(CO)_3Br$ complex, on the other hand, had a significantly reduced efficacy (RSA) compared to parent phenyl vinyl estradiol and compared to their observed RBA values (approximately 0.25-0.4% vs. 4%). Because steric factors should not be significant in modulating binding at the ERα-LBD for the 3-pyridyl analog, the reduced efficacy may represent an influence on the downstream biological response or access to the nucleus. Extension of the pyridyl moiety to the rhenium tricarbonyl coordinated bipyridyl analog produced no further alteration in function, suggesting a similar mode of binding and response modulation for the two derivatives.

In summary, we have demonstrated the preparation new class of rhenium tricarbonyl coordinated ligands for the estrogen receptor through the novel Stille coupling of the 5-bromo-2,2'-bipyridine- $Re(CO)_3$ complex and the vinylstannane. Initial *in vitro* evaluation indicated that although the observed affinity for the initial examples is lower compared to estradiol, it has been demonstrated that *in vitro* binding and *in vivo* activity for this type of 17α-substituted estradiol derivatives can be significantly enhanced by appropriate 11β-substituents, such as methoxy, ethyl or vinyl.[21] Replacement of the rhenium by technetium-99m, the gamma-emitting radionuclide, should then provide a radiopharmaceutical with potential for *in vivo* imaging ER-containing tissues, such as hormone responsive breast cancer. Further studies along those directions are in progress.

Acknowledgments.
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Supporting Information

General Methods. All reagents and solvents were purchased from Aldrich or Fisher Scientific. THF and toluene were distilled from sodium/benzophenone. Many of the reactions were carried out in air although in a few cases, a blanket of N_2 was used. There appears to be no danger of oxidation in these reactions. Tetrahydrofuran was distilled from Na/benzophenone while methanol and other solvents were used without purification. Reactions were monitored by TLC, performed on 0.2 mm silica gel plastic backed sheets containing F-254 indicator. Visualization on TLC was achieved using UV light, iodine vapor and/or phosphomolybdic acid reagent. Column chromatography was performed on an Argonaut Flashmaster using prepacked Isolute silica gel columns. $Re(CO)_5Br$, (1) $[NEt_4]_2[Re(CO)_3Br_3]$ (2) and 3-bromopyridine were prepared by literature procedures. Melting points were determined using an Electrotherm capillary melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded in acetone-d^6 or methanol-d^4 referenced to residual protons in the solvent ($CD_3COCD_2\mathbf{H}$ at 2.05 ppm relative to TMS at 0.0 ppm) or to the ^{13}C in the solvent, $(\mathbf{C}D_3)_2CO$, at 130.7 ppm.. (3) IR spectra were recorded as Nujol mulls on a Mattson Satellite FTIR interfaced with a Digital PC3000 computer. All compounds gave satisfactory elemental analyses, ± 0.4%, (Desert Analytics, Tucson, AZ) unless otherwise stated. 1H-, 13C-, -spectra and elemental analyses are provided.

17α-E-(3-Pyridyl)-vinyl estradiol.

17α-E-tri-n-butylstannylvinyl estradiol (0.50 mmol, 0.293 g) , 3-iodopyridine (1.50 mmol, 0.310 g), dried cesium fluoride (0.40 g), and 25 mg bis (tri-t-butylphosphine)palladium (0) were evacuated and purged with argon four times. Dry dioxane (3 mL) was added, the mixture was sealed under an argon atmosphere and heated at 80°C until the reaction was complete (monitored by TLC). The hot reaction mixture was filtered and the residue was washed with acetone. The filtrate was evaporated to dryness and the product was purified by flash chromatography on silica gel using hexane-ethyl acetate (gradient) as the eluent. The fractions containing pure product were combined and evaporated to yield 34 mg (0.09 mmol, 18% yield). The product was characterized by ^1H-, ^{13}C-NMR, HRMS, and elemental analysis.

Synthesis of 5-Bromo-2,2'-bipyridine$Re(CO)_3Br$

Method A: Reaction of $Re(CO)_5Br$ with 5-bromo2,2'-bipyridine

A slurry of 218 mg (0.54 mmol) $Re(CO)_5Br$ and 107 mg (0.52 mmol) 5-bromobipyridine in 25 mL THF was heated to reflux under nitrogen for 20 h. Solvent was evaporated from the yellow solution under vacuum yielding 258 mg (86% yield) of (5-bromobipyridine)$Re(CO)_3Br$ as a yellow solid. The compound remains unchanged upon heating to 250°C.

Analysis: Calculated for $C_{13}H_7Br_2N_2O_3Re$: 26.77 % C, 1.21 % H, 4.80 % N; Found: 27.07 %C, 1.41 % H, 4.97 % N.

IR (Nujol) ν_{CO} = 2015, 1915, 1894 cm^{-1}

^1H (acetone-d^6): 7.83 t or d (J = 1.2, 7.5 Hz, 1 H), 8.35 t of d (J = 1.8, 8.7 Hz, 1 H), 8.55 dd (2.4, 12 Hz, 1 H), 8.74 d (J = 8.7 Hz, 1 H), 8.79 d (J = 8.1 Hz), 9.14 dd (J = 0.6, 5.4 Hz, 1H), 9.21 d (J = 2.4 Hz, 1 H)

^{13}C (acetone-d^6): 126.12, 126.79, 129.57, 138.86, 141.38, 141.73, 151.02, 151.66, 155.04, 156.53, 180.78

Method B: Reaction of $[NEt_4]_2[Re(CO)_3Br_3]$ with 3-bromobipyridine

A slurry of 395 mg (0.51 mmol) $[NEt_4]_2[Re(CO)_3Br_3]$ and 109 mg (0.52 mmol) 3-bromobipyridine in 25 mL of methanol was refluxed for 18 h, precipitating a yellow solid. After cooling, the precipitate was collected by filtration yielding 205 mg of (3-bromobipyridine)$Re(CO)_3Br$ contaminated by NEt_4Br. Washing the crude product with three 10 mL aliquots of water followed by drying under vacuum yielded 141 mg (48 % yield) of (3-bromobipyridine)$Re(CO)_3Br$. The product is spectroscopically identical to the product isolated from the reaction between $Re(CO)_5Br$ and 3-bromopyridine.

17α-(5-bipyridyl)vinyl estradiol rhenium tricarbonyl complex.

17α,20E)-21-(5-bipyridyl)Re(CO)$_3$Br-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol (EM-1460-3A)

A mixture of CsF (289 mg, 1.89 mM) previously dried at 110 ºC for 24 hours, 3-hydroxy-(17α,20E)- 21-(tri-*n*-butylstannyl)-19-norpregna-1,3,5(10)20-tetraene-17β-ol (150 mg, 0.26mM) and (5-bromobypridine)Re(CO)$_3$Br (152 mg, 0.26 mM) was added to a reaction tube and exchanged four times with argon. Bis(tri-t-butylphosphine) palladium(0) (25 mg, 0.027mM) and dry, degassed 1,4-dioxane (1 mL), prepared by the distillation from sodium/benzophone under argon, were then added to the reaction tube and heated at 70 ºC for 24 hours. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate (50mL), filtered and absorbed onto Florisil (5 g.). The mixture was transferred to a pre-equilibrated column and (17α,20E)-21-(5-bipyridyl)Re(CO)$_3$Br-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol isolated using flash chromatography to give 64 mg((30 % yield) as an amber solid and characterized by ^{1}H,^{13}C-NMR , IR and elemental analysis.[insert data]

Biological Assays.

The methodology for determining estrogenic potency, estrogen receptor binding and stimulation of an estrogen responsive gene, alkaline phosphatase in the Ishikawa cell was performed as we have previously described (4).

Competitive Binding to Rat Cytosolic ER, Human LBD-ERα and Human LBD-ERα ERβ. Binding affinities of the estradiol derivatives relative to E_2 were performed in incubations with the LBD of ERα. in lysates of *Escherichia coli* in which the LBD of human ERα (M_{250}–V_{595})(5) is expressed as described (6) The assay was performed overnight in phosphate buffered saline + 1 mM EDTA at room temperature. The competition for binding of [^{3}H]E_2 to the LBD of the E_2-derivatives in comparison to E_2, relative binding affinity (RBA) was determined over a range of concentrations from 10^{-12} to 10^{-6} M. After incubation, the media is aspirated, the plates are washed 3 times and the receptor bound radioactivity absorbed to the plates are extracted with methanol and counted. The results, as RBAs compared to E_2, of all receptor studies shown in Table xx, are from at least 3 separate experiments performed in duplicate. RBAs represent the ratio of the EC_{50} of E_2 to that of the steroid analog x 100 using the curve fitting program Prism to determine the EC_{50}.

Estrogenic Potency in Ishikawa Cells. The estrogenic potency of the E_2-analogs was determined in an estrogen bioassay, the induction of AlkP in human endometrial adenocarcinoma cells (Ishikawa) grown in 96-well microtiter plates as we have previously described.(7) The cells are grown in phenol red free medium with estrogen depleted (charcoal stripped) bovine serum in the presence or absence of varying amounts of the steroids, across a dose range of at least 6 orders of magnitude. After 3 days, the cells are washed, frozen and thawed, and then incubated with 5 mM p-nitrophenyl phosphate, a chromogenic substrate for the AlkP enzyme, at pH 9.8. To ensure linear enzymatic analysis, the plates are monitored kinetically for the production of p-nitrophenol at 405 nm. For antagonists, the effect (K_i) of each compound tested at a range of 10^{-6} M to 10^{-12} M was measured for the inhibition of the action of 10^{-9} M E_2 (EC_{50} ~ 0.2 nM). Each compound was analyzed in at least 3 separate experiments performed in duplicate. The K_i and RSA (RSA = ratio of $1/EC_{50}$ of the steroid analog to that of E_2 x 100) were determined using the curve fitting program Prism.

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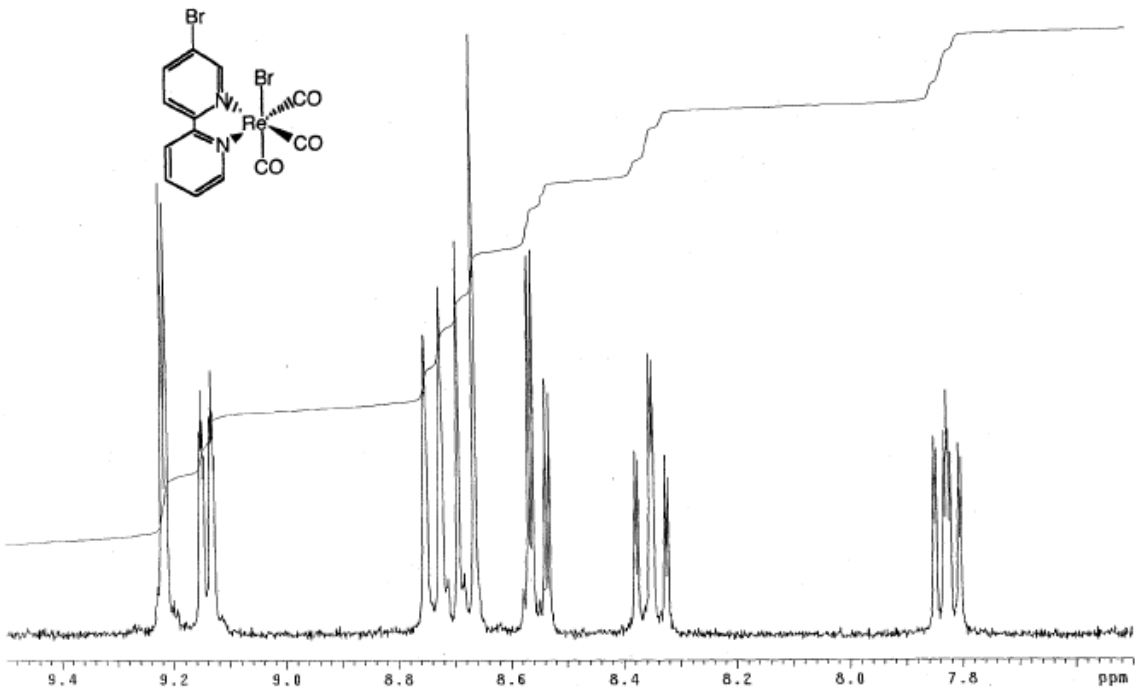

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Synthesis of Benzoyl and Benzyl Conjugates of 17α-E-Phenylvinyl Estradiol and Evaluation as Ligands for the Estrogen Receptor-α Ligand Binding Domain

Robert N. Hanson, Emmitt McCaskill, Edward Hua, Pakamas Tongcharoensirikul, David Labaree and Richard B. Hochberg

Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA 02115
And

Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06520

Abstract:
A series of substituted benzoyl and benzyl derivatives of 17α-E-phenylvinyl estradiol was prepared in good overall yield using a convergent Stille coupling strategy. Biological evaluation using competitive binding assays indicated that all of the compounds were ligands for the ERα and ERβ-LBD, but within a very narrow range of relative binding affinities (RBAs). However, unlike the parent 17α-E-phenylvinyl estradiol, the compounds demonstrated a very low capacity to stimulate the ERα in an Ishikawa cell assay. The results suggest that the compounds may form a complex with the receptor different from that observed for the parent compound.

Introduction: The estrogen receptor (ER) is a member of the nuclear receptor (NR) superfamily, a group of receptor transcription factors that mediate a wide variety of physiological and developmental responses.(1,2) Because inappropriate or over-expression of ER is associated with endocrine disorders, such as breast and endometrial cancer, and osteoporosis, modulation of these ER-regulated responses is a critical clinical objective.(3-5) Recent reviews have highlighted the structure of the ER, including its subtypes, and the general mechanism by which binding of the endogenous ligand initiates the events leading to transcription.(6-10) While many individual steps are involved in the overall estrogenic process, the initial binding of ligand to the unliganded (apo)receptor to form a stable complex remains the key step. Subsequent biological responses are influenced by the receptor conformation induced by this interaction. Based upon this observation, research efforts to characterize that initial step remain important for understanding how the subsequent biological effects are generated. As part of our program to develop steroidal probes of the ligand binding pocket (LBP) of the estrogen receptor (ER), we prepared and evaluated several series of E-17α-(ortho-, meta- and para-substituted phenyl) vinyl estradiols.[11-14] [Figure 1]

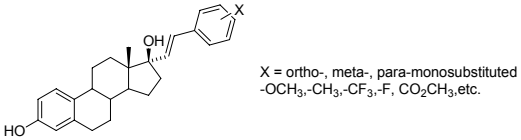
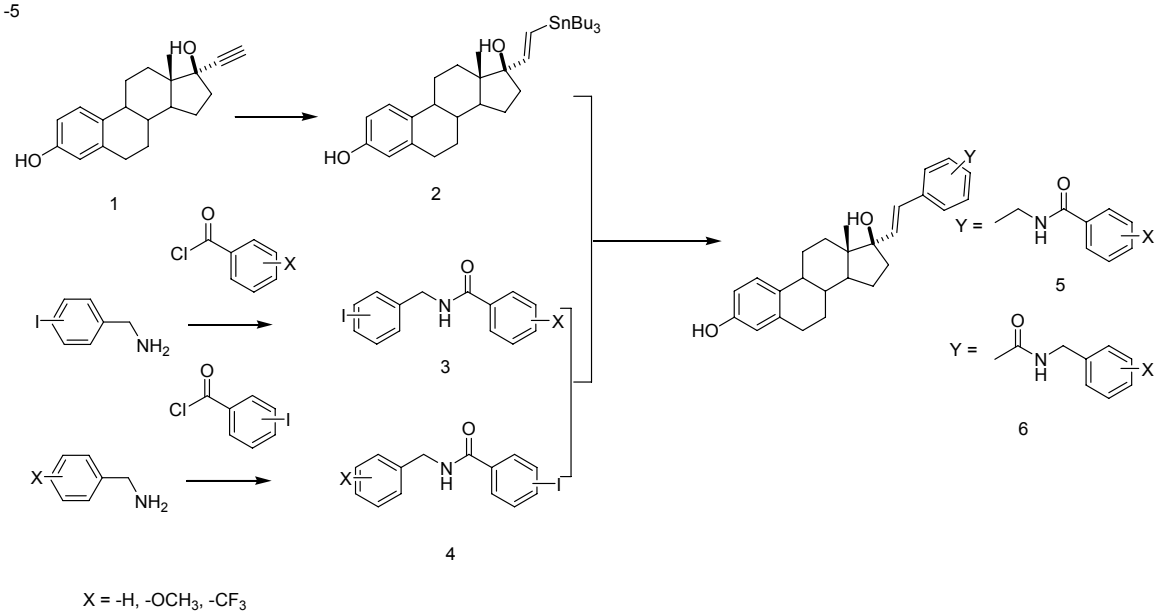


Figure 1. Representative 17α-E-(Subsituted Phenyl)vinyl Estradiols

These compounds, readily accessible via Stille couplings of the requisite aryl iodide/bromide and the E-stannylvinyl estradiol, displayed a range of relative binding affinities (RBAs) compared to estradiol (range <1% to >200%). Regardless of RBA values, all of the compounds expressed full agonist activity in uterotrophic or cellular assays. The derivative possessing the ortho-trifluoromethyl substituent was of particular interest because it displayed the highest binding affinity and greatest in vivo estrogenic activity. Molecular modeling studies, coupled with x-ray crystallography, indicated that the plasticity of the ER-ligand binding domain protein allowed the residues within the ligand binding pocket (LBP) to accommodate the additional steric demands imposed by the 17α-(substituted phenyl)vinyl substituent.[15] The objectives of this current study were to explore the limits to which the protein could undergo adaptation and to determine whether additional steric constraints would induce an antagonist as opposed to agonist conformations and responses.

Chemistry: Our synthetic strategy, shown in Scheme 1, was adapted from our earlier studies. The stannylvinyl estradiol 2 was readily prepared via stannation of ethynyl estradiol 1 using triethyl borane as the radical initiator. The iodoarene coupling partners 3

and 4 were prepared in excellent yields by either (methoxy/trifluoromethyl) benzoylation of the iodobenzylamines or iodobenzoylation of the (methoxy/trifluoromethyl)benzylamines.[16,17] These substitution patterns would allow exploration of the adjacent receptor topology with electron donating and withdrawing groups. Both procedures gave products that were readily purified by recrystallization and characterized. Coupling of the stannylvinyl estradiol and the functionalized iodoarenes 3 and 4 using our standard Stille coupling method gave the desired products 5 and 6 in good overall yield, after flash chromatographic purification. [18]



Scheme 1. Preparation of N-Benzylcarboxamido/-Aminomethylbenzoyl conjugated phenyl vinyl estradiols 5,6.

Biological results: The new compounds were evaluated as ligands for the ERα-and ERβ-LBD using a competitive binding assay and for efficacy using the induction of alkaline phosphatase in Ishikawa cells. [18-20] The results for the binding assay are shown in Table 1. The RBA values are compared to both estradiol (RBA = 100%) and the parent 17α-E-phenylvinyl estradiol (RBA = 7%). As the results show, all of the compounds tested demonstrated significant ER-LBD binding with RBA values ranging from 0.5-10%. The highest RBA value was found for the meta-benzylamine with the terminal 4-methoxybenzoyl group **5h**, while the lowest RBA value was obtained for its ortho benzylamine isomer **5i**. Virtually all of the other compounds had values in the 1.5-7.0% range. There appeared to be virtually no selectivity for ERα-LBD versus ERβ-LBD within this set of compounds as binding ratios were less than 10:1 for either LBD subtype. Evaluation of the compounds as agonists or antagonists in the Ishikawa cell assay indicated that none of the new compounds induced alkaline phosphatase at concentrations below 1 μM. Neither did they block the induction of alkaline phosphatase by estradiol. The parent phenyl vinyl estradiol was a full agonist with a relative stimulatory activity (RSA) of 9% compared to estradiol (RSA = 100%).

Discussion:

Given the molecular dimensions and physicochemical properties of the additional functional group, it is significant that the maximal loss of binding affinity was less than one order of magnitude compared to the phenylvinyl estradiol. The introduction of an additional functionalized benzoyl/benzylamino group into a binding pocket that is as closely bounded as the 17α-position of estradiol was expected to have a greater impact on binding. With the simpler substituted phenyl vinyl estradiols, shifting a trifluoromethyl group from the ortho to meta to para position reduced binding by an order of magnitude.(13) In this present case, introduction of an additional benzene ring, along with the amide linkage and a terminal methoxy/trifluoromethyl moiety, had a minimal effect on the ability of the new ligand to compete with the binding of estradiol at the ER-LBD.

Of equal significance is the observation that the RBA range is narrow and the values are essentially unaffected by the variation within the amide linkage or the substitution pattern on either aryl ring (**5a-i** or **6a-d**). The observation that the range of RBA values is less than one order of magnitude for the variety of substitution patterns is significant when compared to previous series (11-14). Molecular modeling of the ligand-ER-LBD complex based upon the crystal structure obtained with the ortho-trifluoromethylphenyl derivative (15,21) suggested that major remodeling (adaptation) of the protein would be necessary to accommodate the ligand in the "standard" steroidal binding mode. In this mode, the additional ring would have to generate a consensus conformation, regardless of inter-ring linkage and terminal substitution. Because this was improbable, a more plausible explanation required the formation of a substantially different binding mode with the ER-LBD, i.e., one that would accommodate the 17α-substituent in an orientation that did not interact significantly with the residues of the LBP. We suggest that the additional aromatic ring forces the 17α-substituent to fold beneath the steroidal scaffold and that the ligand then binds to the LBP in a rotated conformation, similar to that observed for the ICI-ER-LBD complex.(22) If that were the case, helix-12 may not be able to completely enfold the ligand and the 17α-substituent would be more exposed to the solvent, rather than to the protein residues. In this orientation the interaction of terminal ring with residues of the LBD would be minimized, leading to similar RBA values.

This unfavorable interaction of the conjugated estrogen with the receptor may provide at least a partial explanation for the poor binding and low efficacy of other 17α-conjugated estradiols (refs 23-29). The introduction of large groups, such as nucleosides, taxol derivatives, geldanamycin or metal chelates, prevents them from interacting within the ER-LBD in the same fashion as estradiol, ethynyl estradiol or phenylvinyl estradiol. While a stable complex may form, it is unlikely that it would exhibit the same downstream responses as the smaller ER-targeted ligands.

In summary, we have demonstrated the facile preparation of a novel class of ER ligands and their binding to the ERα/β-LBD, however, the compounds were inactive in the cellular assay. Analysis of the compounds, using molecular modeling, suggests an alternate binding mode for the ER-LBD in which the terminal substituents are more exposed to the solvent than to the internal protein surfaces. Because further modifications of the ligands described in this study would be unlikely to characterize key regions of the ER-LBD or to generate improved therapeutic candidates, we have elected to terminate this aspect of our research program in order to focus on the 11β-position of the steroid scaffold.

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16. **General procedure for preparation of iodobenzoyl and iodobenzyl derivatives .**
    The (2-/3-/4-iodobenzoyl chloride was dissolved in chloroform containing 1.1 equivalents of triethylamine. As solution of substituted benzylamine in chloroform was added dropwise with stirring over 15 min. The precipitate that formed was removed by filtration and the resulting filtrate was washed sequentially with water, 1N hydrochloric acid, 5% sodium carbonate and brine. The organic phase was dried over magnesium sulfate (anhyd.), filtered, evaporated to dryness and recrystallized from alcohol.
    To obtain the corresponding iodobenzyl derivatives, the same procedure was followed, except that the substituted benzoyl chlorides and isomeric iodobenzylamines were used. All new compounds **3** and **4** were characterized by tlc and NMR spectrometry for purity and identity.
17. **General procedure for the Stille coupling with 17α-E-Tri-n-butylstannylvinyl estradiol and the substituted phenyl iodides.**
    To a reaction tube containing (17α -20E)- 21-(tri-*n*-butylstannyl)-19-norpregna-1,3,5(10)20-tetraene-3,17β-diol, **2 ,** a few crystals of 2,6 di-tert-butyl-4-methylphenol and the iodobenzoyl/iodobenzyl derivatives were dried under vacuum for 24 hours then exchanged with argon at least four times. Tetrakis (triphenylphosphine) palladium (0) (0.024 g, 0.02 mmol) and dried degassed toluene (5 mL) were added and heated at 110˚C for 6 – 18 hours. On cooling to room temperature the mixture was transferred to a flask with ethyl acetate (50mL), activated charcoal added, heated to boiling, and filtered through a celite pad. To the filtrate containing the (substituted phenyl) vinyl estradiol derivative, fluorsil (4 – 8 g.) was added and evaporated to dryness. Hexane was then added to the slurry and again evaporated to dryness. The mixture containing the substituted phenyl vinyl estradiol was isolated using flash chromatography and characterized by NMR, ^{13}C- and ^{1}H-, and elemental analysis.
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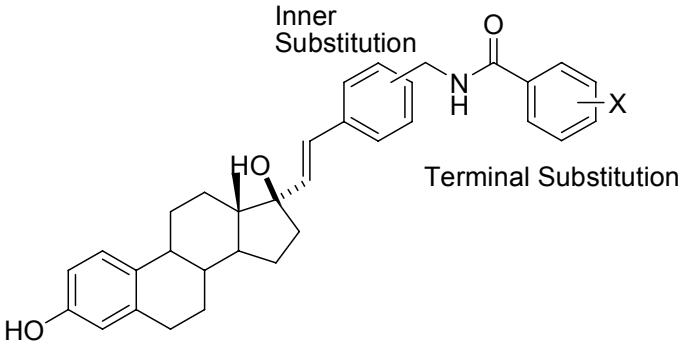

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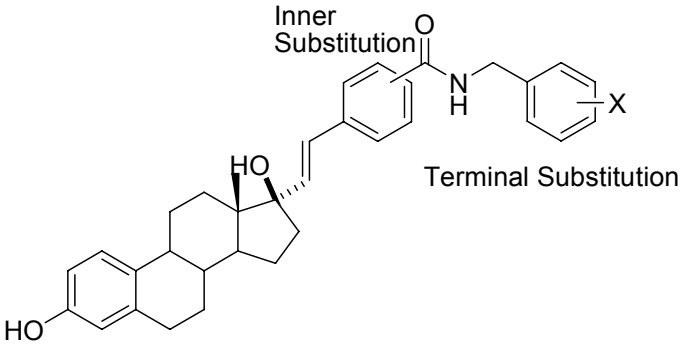

6

Compound	Inner substitution	Outer substitution	RBA ER-alpha (E2= 100)	RBA ER-beta (E2 = 100)
Phenylvinyl	-	-	7.0	7.8
5a	4-	H-	4.2	1.1
5b	4-	4-OCH_3	2.4	3.7
5c	4	3-OCH_3	3.6	3.0
5d	4	2-OCH_3	4.5	5.3
5e	4-	4-CF_3	1.9	1.0
5f	4-	3-CF_3	2.4	2.7
5g	4-	2-CF_3	2.3	3.3
5h	3-	4-OCH_3	10.0	n.d.
5i	2-	4-OCH_3	0.5	n.d.
6a	4-	4-OCH_3	7.9	4.7
6b	4-	2-OCH_3	2.6	2.7
6c	2-	4-OCH_3	2.1	1.0
6d	2-	2-OCH_3	1.4	0.2

Table 1. ER-alpha and ER-beta Relative Binding Affinity (RBA) of Conjugated Phenylvinyl Estradiols 5 and 6

RBA = 100 X [E]/[C] where [E] is the concentration of unlabeled estradiol necessary to reduce the specific binding of tritiated estradiol to the ERα-HBD by 50% and [C] is the concentration of the competitive ligand necessary to reduce specific binding by 50%. The RBA of estradiol is 100% at 25 °C. Curves for ligand and estradiol had correlation coefficients >95%.